SYNTHESIS OF PIGMENT DURING THE RECONSTITUTION OF TUBULARIA [1]

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During the reconstitution of the hydroid, *Tubularia,* a red pigment appears in the region where the new hydranth structures will arise (Loeb, 1891; Driesch, 1900; Morgan, 1901; Stevens, 1901, 1902). This pigment is presumably the carotenoid, astacene, which gives the characteristic red color to the adult hydranth and the pink color to the adult stem (Lönnberg and Hellström, 1931; Karrer and Jucker, 1950; Cohen, 1952). Little is known concerning the origin of the pigment during reconstitution, but previous work has suggested four possibilities (*cf.* Cohen, 1952).

1) The pigment could be synthesized by symbiotic red algae which are believed to occur occasionally in *Tubularia.* This is an appealing possibility because no animals are known with certainty to synthesize carotenoids (*cf.* Karrer and Jucker, 1950).

2) Pigment in the gastrovascular fluid could be taken up differentially by the reconstituting region. Algae or small crustaceans, which are regularly ingested, could provide the source of this pigment, or it could be extruded from cells located at some distance from the forming hydranth.

3) Pigment-containing cells from other regions of the stem could migrate into the hydranth rudiment. This possibility is favored by the observation (Cohen, 1952) that the coenosarc thickens in the regenerating zone and becomes thinner in adjacent regions. Tardent's (1952) finding that the interstitial cells of the ectoderm can migrate extensively also indirectly supports this alternative.

4) Finally, new pigment could be synthesized in the coenosarc, either *in situ* or throughout the stem. If the synthesis were not localized in the reconstituting area, newly formed pigment might then be redistributed according to alternatives 2 or 3.

A study of reconstitution in short segments of stems should permit a choice among the various origins of pigment outlined above. A reconstituting segment becomes completely enveloped by perisarc and does not feed. Although reconstitution will occur in segments of any desired length, the new hydranth actually develops in a region only about two millimeters long. Segments of minimal length are almost wholly involved in hydranth formation. Therefore, by using short segments, it should be possible to determine whether a synthesis of pigment occurs or whether some source of pigment extrinsic to the regenerating segment is essential.

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The following account presents the results of such a study in which both histological and spectrophotometric methods were utilized. The data obtained prove that new pigment is synthesized by the coenosarc of a reconstituting segment.

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Materials and Methods

Colonies of *Tubularia crocea*, furnished periodically by the Supply Department of the Woods Hole Marine Biological Laboratory from August, 1952 to January, 1953, constituted the experimental material. Experiment 1 was begun at the Marine Biological Laboratory and completed at the Arnold Biological Laboratory. For all other experiments the colonies, freshly collected at Woods Hole, were placed in a thermos jug containing filtered sea water at the temperature of local waters and were transported by automobile to the Arnold Biological Laboratory. Experiments were started within six to eight hours after collection since it was found that the animals autotomized their hydranths and began to decay within 24 hours when not in running sea water.

Handling of living material

Translucent, young, and healthy stems with little or no branching were selected for uniformity of cross-sectional diameter and coloration. Stems with a caliber of from 0.5 to 1.0 mm. were cut into segments from 1.5 to 2.0 mm. in length. Six hundred to 1000 such segments were allowed to reconstitute in three or four large finger bowls, each containing about 100 cc. of filtered sea water. In Experiment 1, begun at Woods Hole, the segments reconstituted at room temperature (20-22° C.); in all subsequent experiments, the temperature was kept between 12 and 15° C.

Reconstituting segments were analyzed at each of four arbitrarily designated phases of the regenerative process. Approximately one quarter of the segments was removed from the culture bowls at each of the four phases. In the latter three phases only segments with visible indications of regeneration were selected for analysis. The segments were placed on filter paper and rolled individually to remove any pigmented algae or actinulae larvae which might be adhering to the perisarc. After a period of drying in air at room temperatures which were relatively constant during the experiments, the segments were weighed. The segments of Experiment 1 were weighed one to two hours after removal from the culture bowls; those of Experiment 2, within 20 to 40 minutes after removal. To minimize the variation in wet weight due to evaporation during shorter drying periods, the time interval from the removal of the first segment to the weighing was standardized at 35 minutes in all subsequent experiments. A period of 35 minutes was selected after a preliminary comparison of weights determined after 35 minutes of drying in air, with weights determined after over-night dehydration at 110° C., demonstrated a good correlation between them. During the last 10 minutes of the drying period, the seg-

ments were placed in the analytical balance chamber to permit the atmosphere in the chamber to come to equilibrium.

Spectrophotometric analysis

The amount of pigment in the reconstituting segments, was determined with a Coleman Junior Spectrophotometer. After weighing, the segments in a given phase of regeneration were transferred to a test tube containing 2 cc. of acetone for extraction of the pigment. The segments were ground with the flat end of a glass rod until most of the visible red color had been dissolved (ca. 5 minutes). The mixture was then stored for an extended period at 12° C. (4 to 6 weeks in Experiment 1; two days in Experiments 2–6) to extract any remaining pigment.

In Experiment 1 after acetone extraction, each acetone-extract, plus a 0.5-cc. acetone wash of the residue, was directly diluted to 5 cc. in a volumetric flask; optical densities were then determined. Since all of the optical densities obtained in this experiment were below 0.05 optical density units, a micro-method was employed in all subsequent analyses (Experiments 2–6).

In this procedure, after extraction with acetone, each acetone extract, plus a 0.5-cc. acetone wash of the residue, was transferred to another test tube and evaporated to dryness (overnight vacuum desiccation). The residue of the once-extracted segments was re-extracted for two days with 2 cc. of acetone. This supernate, plus a 0.5-cc. acetone washing of the residue, was desiccated in a similar manner. The first and second desiccated extracts were then taken up separately in acetone delivered from calibrated volumetric micro-pipettes. A 0.3889-cc. micro-pipette was used in Experiment 2; a 0.1565-cc. micro-pipette in Experiments 3–6. The pigment solutions were transferred to cork-stoppered, standardized micro-cuvettes made from No. 5 Pyrex tubing for the Coleman Junior instrument. To minimize evaporation, all dilutions were carried out in the open freezing compartment of a refrigerator, using previously chilled acetone and equipment. Optical densities were then determined.

The maximum absorption of the extracts fell between 460–470 mμ (*cf.* Cohen, 1952); their optical densities were therefore compared at 470 mμ. The absorption values of the first and second extracts were added, and their sum divided by the wet weight of the segments originally extracted. Since the dilutions employed in Experiments 1 and 2 were different from those used in Experiments 3–6, a dilution factor was introduced to make all absorptions directly comparable.

Histological methods

For histological study of pigment distribution, tissues were fixed in a solution of three parts absolute alcohol-one part acetic acid, and were then sectioned in paraffin and stained with iron hematoxylin. Pigment distribution was also examined in squash preparations of living material viewed either with polarized or white light.

The sudanophilia of adult hydranths and of several reconstituting segments was analyzed, using Baker's (1944) method with the following modification: the tissues were fixed for 20 minutes in a solution containing 10 cc. of formalin, 10 cc. of 10% calcium chloride and 80 cc. of filtered sea water. After three rinses in tap water, the pieces were imbedded in 25% gelatine and cut at 10 μ with the freezing microtome; sections were stained with Sudan black B for 8 minutes.

Experimental Results

The phases of reconstitution selected for study are shown diagrammatically in Figures 1a–1d. The *zero point phase* (Fig. 1a) includes pieces selected immediately after the last segments had been amputated at the start of a given experiment; from 0 to 1½ hours had elapsed since the segments had been isolated and most of their

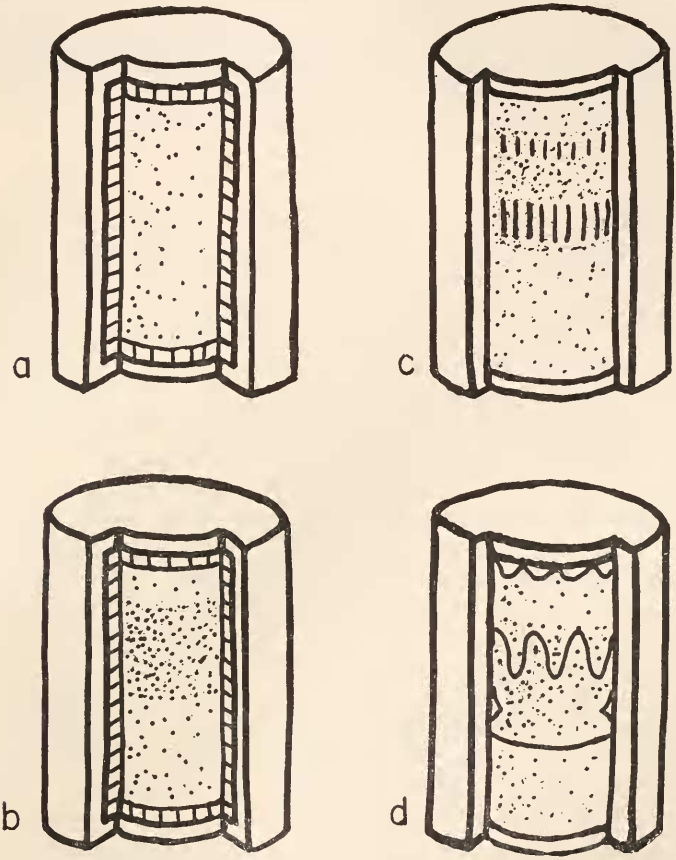

Figure 1. Three dimensional representation of reconstituting *Tubularia* stem segments. The characteristic red pigment distribution is shown by stippling. The clear outer covering represents the perisarc and the hatched layer in a and b, the ectoderm. a, Zero point phase segment showing pigment in the exposed endoderm. b, Condensation phase segment with band of pigment in the future hydranth endoderm. c, Striation phase; the proximal and distal pigmented striations are visible through the intact ectoderm (within the band of pigment). d, Intraperisarcal hydranth phase segment showing the unemerged hydranth within the perisarc.

cut ends had already healed. The first visible indication of reconstitution appears at the *condensation phase* (Fig. 1b) after 34–35 hours at 12–15° C. At this time, a circumferential band of pinkish-red pigment, some 0.5 to 2 mm. in length, becomes localized in the future distal half of the segment. After approximately 40–45 hours

of reconstitution, the *striation phase* (Fig. 1c) can be distinguished. This phase is characterized by two parallel rows of well-defined, longitudinal red striations which have developed in the pigmented band. The final, *intraperisarcal hydranth phase* (Fig. 1d), which appears after about 60–62 hours, features a constriction separating the newly formed hydranth from the coenosarc.

Origin of pigment

As pointed out in the Introduction, the work of Cohen (1952) has suggested that the externally visible reddening which occurs during reconstitution in the area of hydranth formation is due to one of four possibilities. The following account considers each of these alternatives in turn.

Role of red algae

Ceramium and other red algae which might contribute to the reddening of the hydranth-forming region, were sought in several squash preparations of living material as well as in fixed sections of 7 adult *Tubularia* and 8 segments in different phases of reconstitution. Both solitary and colonial forms of *Ceramium,* as well as *Achrochaetium* and *Rhodochorton,* have been observed in *Tubularia,* either in the endoderm or between the endoderm and the ectoderm (W.R. Taylor, personal communication). The individual algal cell is small, rectangular in shape, and contains a readily distinguishable red pigment which is not removed by ordinary histological procedures. No such cells were demonstrable in the coenosarc. Cohen (personal communication) has already observed that only occasional *Tubularia* are infested with these organisms. His results, combined with those obtained in the present study, indicate that red algae are not indispensably related to pigment formation.

Further support for this conclusion was obtained in experiments with $CuSO_4$, a compound which has been widely used as a specific poison for fresh water and marine algae (Moore and Kellerman, 1905; Domogalla, 1926; Prescott, 1938). In experiment 4, the colonies were dipped for about two minutes into a solution of 5% $CuSO_4$ in sea water immediately prior to the amputation of segments. The bottom of the bowl to which the colonies were transferred was covered with dead algae within one to two hours. Stem segments cut from these colonies nevertheless regenerated with the normal reddening of the hydranth anlagen. Identical results were obtained in Experiment 5, in which the segments were amputated 10 days after the colonies had been treated with 5% $CuSO_4$. This observation also ruled out the possibility that extra-perisarcal algal contamination plays a role in pigment formation.

Concentrating of pigment formed elsewhere

According to this view, pigment circulating in the gastro-vascular fluid is taken up by endodermal cells of the reconstituting region. This pigment would be derived either from ingested substances or from cells lying at some distance from the reconstituting region.

This alternative is ruled out by the fact that in the present experiments, reconstitution occurred in short segments which were entirely enveloped by perisarc. They were therefore completely isolated systems with respect to acquisition of new pigment from the rest of the stem, or from feeding activities.

Migration of pigmented cells

This alternative suggests that pigmented cells may migrate from a distance into the reconstituting region. Extensive cell migration cannot be critically important, however, because pigment accumulates normally during the reconstitution of short segments which are isolated from the rest of the stem.

Localized synthesis of pigment

The above observations show that the reddening in the hydranth anlagen cannot be attributed to red algae, ingested materials, or a concentrating of pigment already present. Therefore, the reddening must be due to an increase of pigment within the

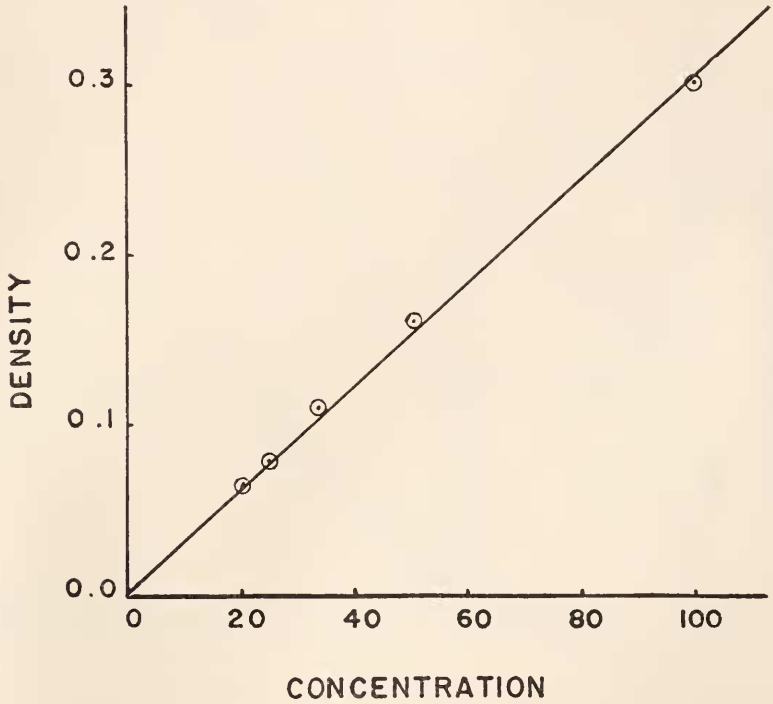

FIGURE 2. Comparison of concentration and optical density of red pigment extracts. Density is plotted along the ordinate as optical density units and concentration along the abscissa as per cent of the most concentrated.

reconstituting segment, *i.e.*, a synthesis of pigment by the coenosarc. Since microscopic observation is not adequate to provide direct evidence on this point, the pigment content of segments at successive phases of the reconstitutive process was determined by a spectrophotometric assay.

Evaluation of method. In order to determine the validity of using optical density as a direct measure of pigment concentration, and the reliability of using the densities obtained in the lower range of the spectrophotometer, the following preliminary determination was made.

A concentrated acetone extract of adult hydranths was prepared and diluted: 1 : 2, 1 : 3, 1 : 4, and 1 : 5. The absorption values for the concentrated extract and the four dilutions were plotted against dilution expressed as per cent of the most concentrated. The resulting curve (Fig. 2) demonstrated that for optical density values of 0.05 or above, a linear relation exists between concentration and density.

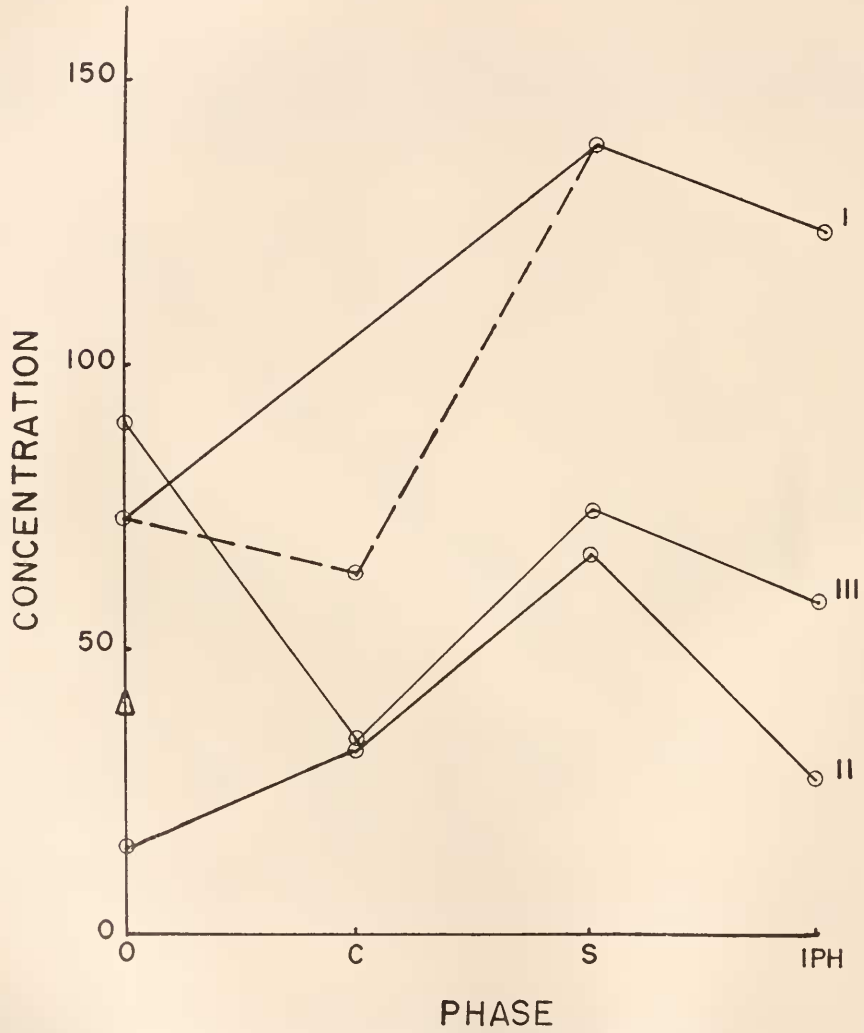

FIGURE 3. Changes in the concentration of red pigment during reconstitution. Concentration, expressed as optical density units per mg. wet weight of segments $\times 10^{-4}$, is plotted against phase of reconstitution. O, C, S, and IPH represent the zero point, condensation, striation, and intraperisarcal hydranth phases, respectively. The optical densities were measured at 470 mμ. The triangle denotes the concentration of red pigment in segments from Experiment 6 which failed to reconstitute after four days of culturing. Curves I, II, and III represent Experiments 1, 2, and 6, respectively.

Several of the values subsequently obtained with the experimental material fell between 0.01 and 0.05 optical density units. These values appear to be trustworthy, however, since curves plotted from them were corroborated in other cases where higher values were obtained.

Spectrophotometric results. The data from Experiments 1, 2, and 6 are shown in Figure 3 (curves I, II, and III, respectively), plotted as pigment concentration per unit wet weight against phase of reconstitution. The values on which these curves are based appear in Table I.

In general, the curves show a marked increase in the amount of pigment from the zero point phase to the striation phase, followed by a moderate decrease from the striation phase to the intraperisarcal hydranth phase. Although the conditions un-

TABLE I

Red pigment extracts

Experiment	Stage*	Wet weight mg.	Optical density	Optical density/ mg. wet weight $\times 10^{-4}$	Optical density/ mg. wet weight at dilution of Exps. 3–6 $\times 10^{-4}$
1	O	44.4	0.012	2.3	73.2
	C	44.8	0.010	2.0	63.7
	S	74.1	0.038	4.3	137.2
	IPH	89.8	0.039	3.9	124.2
2	O	44.8	0.028	6.3	15.8
	C	45.6	0.060	13.2	33.0
	S	35.2	0.096	27.3	68.3
	IPH	36.6	0.040	10.9	27.3
6	O	66.8	0.580	86.8	86.8
	C	96.4	0.327	33.9	33.9
	S	34.0	0.252	74.1	74.1
	IPH	40.2	0.234	58.2	58.2
	N–R	33.6	0.128	41.7	41.7

* O represents the zero point phase; C, the condensation phase; S, the striation phase; IPH, the intraperisarcal hydranth phase; and N–R, the non-reconstituted phase segments in Experiment 3.

der which the data were obtained preclude precise quantitative statements about the absolute increase in pigment, the curves do reveal the approximate range of this increase. They show: 1) that the concentration of pigment increases up to the development of the third phase of reconstitution to values some two- to four-fold above that of the base level of the zero point phase, and 2) that pigment decreases during the development of the last phase to a final value some one- to three-fold greater than that of the base level.

The upward displacement of curve I is probably due to the large dilution factor required to make the original optical density values directly comparable with those comprising curve III (see Materials and Methods). The first three points of curve I are interconnected with dotted lines since the data for the condensation phase in this experiment were obtained from an extract which was recovered after some spill-

age. The second point therefore represents at least a minimum concentration for this phase but the actual value should be higher.

The extract of the zero point phase in Experiment 6 (curve III) was observed to be redder than those of the other phases before desiccation. This indicated that the aberrantly high pigment concentration found for this phase was not due to some procedural error during dilution or spectrophotometry. The high value may be due to either: 1) a non-random selection of more highly pigmented segments to represent the zero point phase, or 2) a decrease, after the removal of zero point phase segments, in the number of pigmented larvae or algae adhering to the remaining segments. Statistically, the first alternative appears unlikely. But there is no direct evidence favoring the second and the issue must be left undecided.

Overlooking the problematical high zero point value of curve III, the graphs in Figure 3 demonstrate that the reconstituting segments gained pigment during the development of the second and third phases and lost pigment before the last phase. These findings are indirectly supported by the observation that there is a much smaller increase in pigment content in non-reconstituting segments. Regeneration of all the segments in a culture bowl never occurred. Therefore, many non-reconstituted segments were available for analysis. The pigment concentration was measured for approximately 200 segments (from the lot used for Experiment 6, curve III) which had not reconstituted after four days. The extract of these segments had an adjusted density value (triangle in Fig. 3) somewhat below that of the aberrant zero point phase of curve III but above the more reliable zero point level predicted both by curve II and the slope of curve I. On the basis of this limited evidence, it is possible that a small increase in pigment content has occurred but in any event the increase amounts to less than half of that exhibited by reconstituting segments.

Taken as a whole, the results of these experiments show that there is an increase in total pigment content of short reconstituting segments. Since the new pigment is not derived from extraneous sources, the coenosarc of the segment must synthesize pigment during the regeneration of a hydranth.

The distribution of pigment. To obtain information about the site of synthesis within the segment, the distribution of pigment in adult and reconstituting individuals was studied histologically. While these studies did not provide a definitive solution, they did reveal some pertinent facts about the form and the location in which pigment occurs. In general, two forms of pigment can be identified: 1) large (2.6–10.3 μ) granules, irregular in shape and red in color when viewed in white light, but appearing as elongate birefringent crystals when seen in polarized light, and 2) minute ($< 1\ \mu$) anisotropic crystals, white and luminescent in polarized light and dark red in white light.

Large pigment granules circulate passively in the gastro-vascular fluid of adult, living *Tubularia;* the large granules also occur intracellularly in the endoderm. In the stem, pigment granules are evident particularly in large, oval cells which are sparsely scattered through the endodermal ridges and the rest of the endoderm. Pigmented material occurs in all endodermal cells of the hypostome and is so concentrated that it is not possible to distinguish individual granules. Presumably, some of the pigment in this region is also in granular form. Since the endoderm phagocytizes pigment from the circulation (Cohen, 1952), the large intracellular granules may have this origin.

Other endodermal cells which do not contain the pigment granules, as well as the heavily colored cells of the hypostome, are filled with small, anisotropic pigment crystals. In each such cell, the crystals are homogeneously distributed and are present in large numbers. Presumably, they account for most of the gross red coloration of *Tubularia*.

Stevens (1901) also described two forms of pigment: 1) translucent, yellowish-red (1 to 8.5 μ) "plasmatic granules" which are insoluble in alcohol, and 2) small, irregular grains of red pigment which are soluble in alcohol. In some endodermal cells, Stevens found both types occurring together; in other cases, only the small grains were present. The large red granules, observed in the present investigation, appear to correspond with Stevens' "plasmatic granules"; the minute anisotropic crystals are probably identical to her irregular grains.

During reconstitution, the intracellular pigment granules appear to remain constant in number. The reddening of the hydranth anlagen is therefore probably due to an increase in concentration of the pigment crystals. It is not clear, however, whether the pigment crystals are formed *in situ* or whether there is a general synthesis in all endodermal cells of the reconstituting segment followed by a concentration of pigment or pigmented cells in the reddening area (see Introduction, alternative 4). Only further study can answer this question.

Distribution and concentration of sudanophilic substances

During the course of the experiments just described, attention was drawn to the possibility that lipid substances might be related to reconstitution. A liquid was noticed which adhered to the sides of the test tubes containing the desiccates of the first pigment extracts of Experiments 2–6. The liquid could not be evaporated by vacuum desiccation for two days. The desiccates of the zero point phases contained the largest amounts of this liquid (presumably lipid), while those of the three subsequent phases contained progressively smaller amounts. This observation prompted a histochemical analysis of the sudanophilia of reconstituting segments. The variations in the degree of sudanophilia which were detected in this analysis could not be related to the relative amounts of liquid in the desiccates. But they provided some important additional information about the reconstitution process. The methods used and the results obtained were as follows.

Studies were made on 4 to 8 sample sections (stained with Sudan black B) of three adult hydranths and stems, and of one or two segments in each phase of reconstitution from Experiments 3 and 6. The degree of sudanophilia was estimated by averaging arbitrarily assigned values for the intensity of coloration of the various regions. Five categories (0, + 1, + 2, +3, and + 4) of sudanophilia, representing a range from the least to the most intense coloration, were distinguished. In most cases it was possible to assign a value of 0, + 2, or + 4; the intermediate values of + 1 and + 3 were required only in a few instances. The average level of sudanophilia in each of the various regions of each phase was plotted against the phase of reconstitution (Fig. 4).

Adult hydranths and stems exhibit two distinct levels of sudanophilia. The endodermal cells of the hypostome are intensely colored (+ 4) while the stem endoderm contains smaller, diffusely distributed sudanophilic globules. The degree of coloration is similar (+ 2) in the endodermal ridges, in the endoderm subjacent

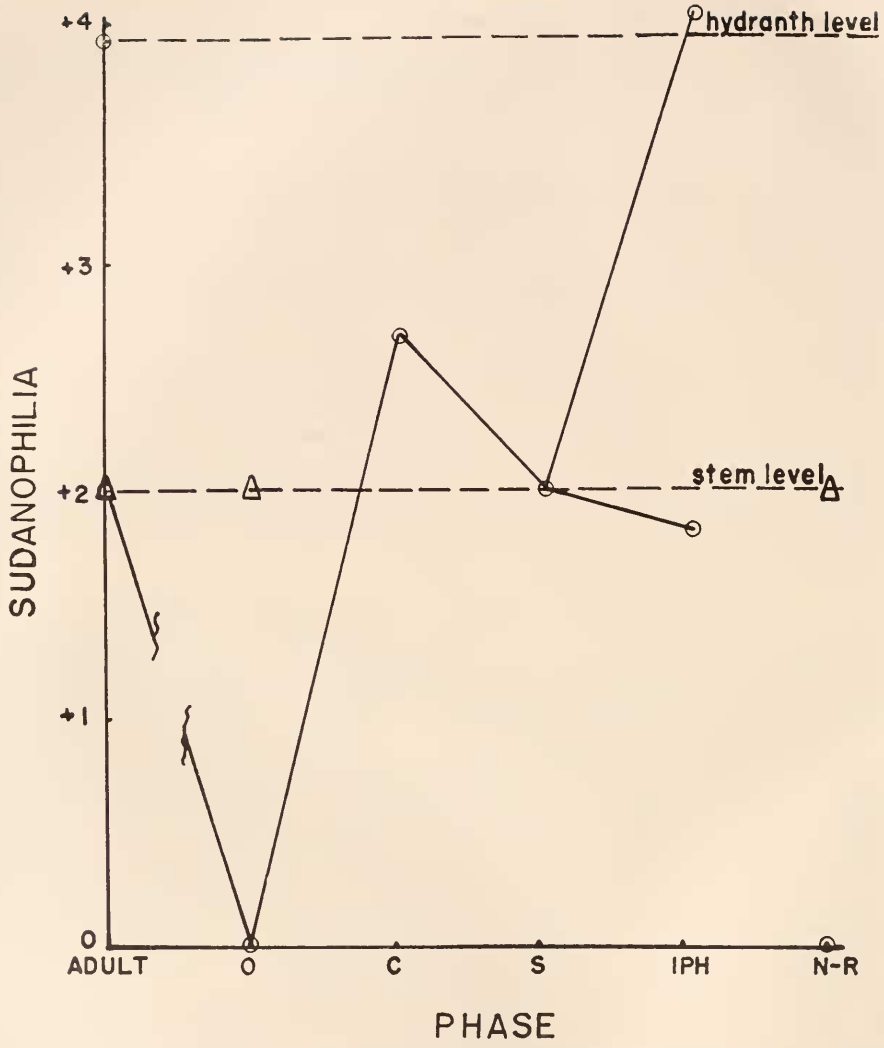

FIGURE 4. Changes in the degree of sudanophilia during reconstitution. Sudanophilia is plotted as arbitrarily designated levels of staining intensity (see text) against phase of reconstitution. N–R represents segments which had failed to reconstitute after four days of culturing; other abbreviations are as in Figure 3. Circles indicate values for the circumferential endoderm; the triangles, those for the endodermal ridges and their subjacent endoderm. The curve from the adult to the zero point phase is broken because the adults were not examined at a time in sequence with the regeneration process.

to the ridges, and in the rest of the endoderm (except for the oval cells which are sudanophobic). The pigment crystals are found in close association with some of the sudanophilic globules.

Two levels of sudanophilia also characterize the endoderm of the zero point phase segments. The cells of the endodermal ridges, which are intact only in some

instances, and the subjacent endoderm, maintain a + 2 level of sudanophilia. The rest of the endoderm has dropped to a zero level. The endodermal ridges in the reconstituting end, and in some cases in both ends, break down before the appearance of the condensation phase (Morgan, 1901; Stevens, 1901, 1902); in this phase, the sudanophilia of other endodermal cells has increased to approximately a + 2 level. There is no significant subsequent change in endodermal sudanophilia up to the striation phase. Thereafter, however, a differential distribution of sudanophilic substances is evident. In the intraperisarcal hydranth phase the + 2 intensity of sudanophilia is maintained by the presumptive endoderm of the stem, whereas the hydranth anlage shows an increase to the + 4 level characteristic of the endoderm of the adult hydranth.

Segments which failed to reconstitute even after four days of culturing provide evidence that the disintegration of the endodermal ridges may be an important link in the reconstitutive process. With the exception of their endodermal ridges, which remain intact in every instance, these non-reconstituted segments are similar to the zero point segments. The sudanophilia of their ridges is + 2; that of their circumferential endoderm is 0.

Aberrant pigment extracts

Absorption spectra of all the previously described red pigment extracts (Experiments 1, 2, and 6) contained a single absorption maximum between 460–470 mμ. However, a straw-yellow solution with a single absorption maximum between 402–410 mμ, was obtained when extracts were prepared of the reconstituting segments in three other experiments (Experiments 3, 4, and 5). The procedures followed in these experiments were identical with the following important exceptions. 1) The segments of Experiment 3 were dehydrated at 110° C. overnight for dry weight determinations before extraction. 2) The colonies used for Experiment 4 were freed of algae by dipping them into a solution of 5% $CuSO_4$ in sea water immediately before segments were cut from the stems. 3) The colonies used for Experiment 5 were immersed in 5% $CuSO_4$ 10 days before segments were amputated. During the 10-day interval between treatment and amputation, the colonies were kept in filtered sea water at 12 to 15° C.; the sea water was changed every one or two days. The hydranths autotomized after the first day; reconstitution followed in the ensuing two to three days.

In these three experiments, reconstitution occurred with the normal reddening of the hydranth anlagen. The yellow extracts of Experiment 3 were obtained from segments which were heated after removal from the culture bowls. Segments from the copper-treated colonies appeared red in life but yielded yellow pigment when extracted.

The pattern of changes in the concentration of the straw-yellow pigment during reconstitution of the heat- and copper-treated segments varied from one experiment to another and no interpretation of them will be attempted. However, the possible significance of the yellow pigment will be brought out in the Discussion.

Discussion

Origin of pigment. Short segments of *Tubularia* stems, which are free of red algae and isolated from extrinsic sources of pigment, develop the characteristic red-

dening of the hydranth-forming regions. A spectrophotometric assay has demonstrated that the total amount of pigment increases markedly in these reconstituting segments. These observations provide cogent evidence that the coenosarc of a regenerating segment synthesizes pigment. The synthesis, the nature of which is unknown, may occur throughout the whole segment or only in the hydranth-forming region.

If synthesis occurs in the entire segment, there must be a subsequent concentrating of pigment in the presumptive hydranth. The results of the present experiments offer no evidence on this point. Cohen (1952), however, has already presented evidence indicating that some re-allocation of pigment occurs during reconstitution. Using an accidentally discovered yellow form of *Tubularia,* Cohen fused 5-mm. segments of yellow stems with similar segments cut from the common red form. Subsequently, he found red pigment in the endoderm of the reconstituting "yellow" ends; these red particles gave the "yellow" hydranth a yellow-orange color. In addition, he noted that carbon particles injected into the gastrovascular fluid of a red segment were taken up differentially by the same endodermal cells which later constituted the red striations. From these observations, Cohen inferred that the linear arrangement of pigment in the primordia of the tentacles is due to a differential phagocytic activity of special endodermal cells.

Cohen's findings demonstrate that pigment already present in a stem segment can be redistributed during reconstitution. Presumably, this redistribution could occur as readily in the 2-mm. segments studied here as it did in Cohen's longer (5-mm.) segments. In a short segment, however, only a relatively small amount of pigment is present initially and synthesis of new pigment probably accounts for most of the reddening of the hydranth anlage. In long stems, containing relatively more pigment initially, re-allocation of the original stores could be a more important factor in the hydranth reddening. Therefore, it is tempting to suggest that the amount of pigment already available, either in extra- or intracellular locations, may influence the amount of pigment which will be synthesized. In this view, longer segments which contain more pigment when isolated should synthesize less pigment during reconstitution. This hypothesis could readily be tested experimentally by comparing the amount of pigment synthesized per unit wet weight of tissue in long and short reconstituting segments.

Morphogenetic significance of pigment. Previous workers have held several different opinions about the functional significance of the pigment in reconstituting *Tubularia* (see Cohen, 1952, for literature). The present work still does not permit a definitive statement but one of the following two alternatives is regarded as likely.

1) The consistent decline in the concentration of red pigment before the last phase of reconstitution (see Fig. 3), suggests that pigment or pigment-associated substances (protein, see Cohen, 1952) might be utilized in or during morphogenesis. Since the amount of tissue remained constant in the isolated segments, this decrease is a true decline in pigment concentration. There also cannot be any loss of pigment by extrusion as described by Stevens (1901, 1902) and Godlewski (1904), since the intraperisarcal hydranths were still enveloped by perisarc. Therefore, a mechanism for the breakdown of pigment may be associated with some morphogenetic activity.

2) On the other hand, the altered pigmentation—as well as the increased sudanophilia (see below)—may be merely a visible by-product of synthetic activities. If this is true, then the pigment is not causally related to morphogenesis.

Changes in sudanophilia. Reconstitution is also accompanied by marked changes in sudanophilia. Following amputation, the circumferential endoderm loses most of its sudanophilia. Concomitantly, the disintegrating endodermal ridges presumably liberate sudanophilic globules to the circulation (*cf.* the translucent globules of Stevens, 1901, 1902, and Hargitt, 1903). During reconstitution, the sudanophilia of the circumferential endoderm is gradually recovered until; at the intraperisarcal hydranth phase, levels of sudanophilia characteristic of the adult hydranth and stem are attained. In contrast, non-reconstituting segments undergo only one of these changes, namely, a decline in the sudanophilia of the circumferential endoderm.

The increase in sudanophilia of circumferential endoderm during reconstitution may result either from intracellular synthesis of new lipids, or from phagocytosis of globules previously released into the circulation—or from both. There is at present no evidence to permit a decision among these alternatives.

The relation of sudanophilia to morphogenesis is also obscure. Sudanophilic substances are increasing in the hydranth endoderm during the terminal phase of reconstitution, that is, at a time when total pigment concentration is declining. The significance of the association of these events in time is uncertain but further investigation is clearly indicated.

Disintegration of endodermal ridges. The breakdown of the endodermal ridges in reconstituting segments has been described frequently (Morgan, 1901; Stevens, 1901, 1902; Cohen, 1952). The present investigation has both confirmed these previous observations and revealed that disintegration of the ridges does not occur in segments which fail to reconstitute. These facts suggest that the breakdown of the endodermal ridges may be an important link in the reconstitutive process.

Yellow pigment extracts. The straw-yellow extracts obtained following heat or copper treatment could be due to one of the following: 1) the unmasking of a naturally occurring yellow pigment, or 2) the production of an isomeric or otherwise chemically altered form of the red pigment. There are no data which directly confirm or deny the first possibility, but evidence favoring the second has been reported by Willstaedt (1934). This investigator found that the carotenoid, astacene (presumably the red pigment in *Tubularia;* Lönnberg and Hellström, 1931 and Cohen, 1952), can be reduced by zinc dust in acetic acid-pyridine solution to give a light yellow derivative. Cohen's (1952) discovery that naturally occurring colonies of *Tubularia* may contain individuals ranging in color from red to yellow may also indicate that one type of pigment can be converted into the other.

Cohen proposes that the naturally-yellow *Tubularia* is a biochemical mutant of the red form. If this is true, and if the natural and experimentally produced yellow pigment are identical, then the heat and copper treatments have produced a phenocopy. A spectrophotometric analysis could readily decide whether or not the two yellow pigments are the same.

Summary

1. The origin of the red pigment in the hydranth-forming region of reconstituting stem segments of *Tubularia* has been investigated histologically and spectrophotometrically.

2. Short (1.5 to 2 mm.), perisarc-enclosed segments, isolated from extrinsic sources of pigment (*e.g.,* ingested materials or other non-regenerating regions of the stem), reconstitute with normal reddening.

3. Histological observations have failed to demonstrate the presence of red algae within the coenosarc of *Tubularia.* Therefore, red algae do not play a critical role in the increased coloration.

4. A spectrophotometric assay of the pigment concentration in short segments at successive intervals in the reconstitutive process has provided evidence that the total amount of pigment increases during regeneration. It is concluded that the coenosarc of regenerating segments synthesizes new pigment. This finding is of interest because the pigment is presumably a carotenoid and because no conclusive evidence has previously been presented that animals synthesize carotenoids.

5. Microscopically, pigment is found in two forms: 1) large, irregular and birefringent granules sparsely distributed in the gastrovascular fluid and in certain endodermal cells, and 2) minute anisotropic crystals occurring in large numbers in the endoderm. The reddening of the hydranth anlagen is presumably due to an increase in the number of pigment crystals.

6. During reconstitution, sudanophilic substances increase markedly in the presumptive hydranth endoderm. As the distal coenosarc endoderm transforms into hydranth endoderm, it develops the characteristic adult level of sudanophilia.

7. The possible morphogenetic roles of pigment and sudanophilic materials during reconstitution are discussed. While there is no certain relation of these substances to the morphogenetic process, further analyses are suggested.

8. The breakdown of the endodermal ridges in normally reconstituting segments, and the lack of endodermal ridge breakdown in non-reconstituting segments, indicate a possible role of these structures in the regenerative process.

9. A straw-yellow pigment, chemically different from the characteristic red pigment, is obtained when reconstituting segments are treated with heat or copper sulfate. This yellow pigment may be similar to that occurring occasionally in natural populations of *Tubularia.*

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